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## New insights in optimizing treatment and the role of cancer stem cells in esophageal cancer

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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2014

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Honing, J. (2014). *New insights in optimizing treatment and the role of cancer stem cells in esophageal cancer*. [Thesis fully internal (DIV), University of Groningen]. [S.n.].

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# Chapter 1

## **General introduction**

In the Netherlands the overall incidence of cancer is still increasing with approximately 40% in the last decade (1). Although technical and therapeutic options are expanding, the overall 5-year survival rate in curative treatable patients has increased with only 9%, from 47% to 56% (2), making cancer a huge burden on patients and society. In alignment with these numbers, the incidence of esophageal cancer has also increased steadily over the last decade from around 1900 patients per year to a current number of around 2500 esophageal cancer patients (3,4). These numbers are equal to other countries in the Western world (5,6). The majority of patients have the histological subtype esophageal adenocarcinoma (EAC), while esophageal squamous cell carcinoma (ESCC) more often occurs in Asian countries (5,7,8). In both types chronic irritation and inflammation seem to be important inducers of malignant transformation. In ESCC, alcohol consumption and tobacco use are the two main known risk factors (9,10), while in EAC, gastro-esophageal reflux disease (GERD) is the major risk factor (11). Other risk factors for EAC are sex, race and obesity, as EAC commonly occurs in white men (7,12), and is associated with an increased Body Mass Index (13). GERD is also known to be an important contributor to the development of Barrett's esophagus (BE), the precursor lesion of EAC (14). BE is a metaplastic lesion in which the normal squamous cell esophageal epithelium is partially replaced by a columnar epithelium which may progress to dysplasia and eventually EAC. Despite being a known precursor lesion, patients diagnosed with BE have a low risk of progressing towards EAC; per year, rates of 0.12-0.85% have been reported (15-17). Patients with BE receive a regular endoscopy scheme, as today we have no other tools yet to predict which patient will progress towards cancer.

After being diagnosed with esophageal cancer, patients are staged according to the current TNM 7th classification using endoscopic ultrasound (EUS), Computed Tomography (CT) scan and an 18F-Fluorodeoxyglucose Positron Emission Tomography (FDG-PET) or PET-CT scan (18). Standard treatment with curative intent consists of neoadjuvant chemoradiation (CRT) followed by a transthoracic esophagectomy (19). The Dutch CROSS trial showed a significant benefit of adding neoadjuvant CRT, consisting of carboplatin/paclitaxel with 41.4Gy, to surgery increasing the median overall survival up to 49.4 months compared to 24.0 months in patients with surgery alone (20). In 29% of patients treated with neoadjuvant CRT a pathologic complete response (pCR) was achieved, but also 18% showed little or no response to CRT. A complete response to neoadjuvant CRT is associated with a better survival in EC patients (21), but it is also known that even pathological complete responders may relapse early within one year, raising questions about

which factors predict these more aggressive tumors.

In patients who are not eligible for surgery, for instance due to tumors which are closely related with- or tethered to vital structures or due to severe comorbidity, definitive chemoradiation (dCRT) or radiotherapy (dRT) is the recommended standard curative treatment (22).

The main causes of poor prognosis of esophageal cancer patients are the tumor extent due to a late presentation of disease, early dissemination and a high recurrence rate. Therefore we need a better understanding of esophageal carcinogenesis and more insight in what drives its progression and recurrence. The Cancer Stem Cell (CSC) model could be helpful in achieving a better understanding of esophageal oncogenesis, which can be important to improve current therapy and to identify novel prognostic markers. This model proposes architecture of the tumor in which a subgroup of tumor cells has stem cell (SC) characteristics, particularly self-renewal and multilineage differentiation capacity, and has enhanced tumorigenic ability compared to the bulk tumor cells (23). The first study was done in leukaemia where CD34+/CD38- tumor cells derived from acute myeloid leukaemia patients had SC characteristics (24). In solid cancers, studies on CSCs have been done in various tumor types, including glioblastoma, breast and colon cancer (25-27).

CSCs are associated with high tumorigenicity and chemoresistance and could provide a possible explanation for current treatment failure and disease relapse. An important aspect of CSCs is their ability to escape cell death induced by conventional chemo- or radiotherapy for example by multidrug resistance due to up-regulation of cellular efflux pumps, quiescence and/or enhanced activation of DNA damage repair (28-30). Similar to normal SCs, CSCs are believed to have more effective DNA damage repair mechanisms that results in resistance to DNA-damaging treatments such as chemo-and radiotherapy. Evidence for this notion was provided in glioblastoma, where CSCs marked by CD133 were shown to repair DNA damage more actively by activating the DNA damage checkpoint (31). Another feature of CSCs is multidrug resistance as a result of the presence of ATP binding cassette (ABC) drug transporters. ABC drug transporters are higher expressed and active in SCs and CSCs, and this characteristic is used in the Hoechst efflux assay in which these cells can be identified as a population with low Hoechst intensity, known as the side population (32). Quiescence and slow cell division is considered a third feature of how CSCs can escape chemotherapeutics, since therapy-induced DNA damage and cell death manifests mostly during mitosis (30,33).

Besides resistance against current therapeutic options, CSCs have also been implicated in metastasis and the subsequent development of secondary tumors, possibly through epithelial to mesenchymal transition (EMT). EMT is a process where tumor cells can acquire a more mesenchymal state, which facilitates tumor cell migration and invasion and metastasis (34). In the epithelial state, cells express epithelial markers such as the membrane bound cell adhesion protein E-cadherin. During the conversion to a more mesenchymal state cells lose E-cadherin and gain expression of mesenchymal markers such as vimentin and fibronectin. Migrating cancer stem cells (MCSCs) have been proposed to have both features of a stemness and EMT phenotype and this subpopulation may be a driving factor in the development of metastases (35-37). According to this concept, MCSCs reside on the invasive front of the tumor and through EMT can disseminate from the tumor. As they have stemness features these migrating tumor cells could populate distant sites where they eventually form metastatic colonies. When taking together the above mentioned highly malignant properties of CSCs, it is easy to imagine that therapies that are effective in reducing the tumor load in patients, but fail to eradicate the CSCs fraction, will not be effective and result in relapse of disease.

Currently there is a strong interest in developing targeted agents against pathways that drive CSCs. For example, the Wnt and Hedgehog (Hh) pathways were found to be important in the maintenance of CSCs in various tumor types, including gastrointestinal cancers(30). Essential in the canonical Wnt-pathway is  $\beta$ -catenin, which in the inactivated state is degraded by a complex consisting of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), Axin1 and Axin2, casein kinase 1 (CK1) and Adenoma Polyposis Coli (APC). Wnt ligands that activate Frizzled receptors cause the disruption of this degradation complex, leading to  $\beta$ -catenin accumulation in the cytoplasm and subsequent translocation to the nucleus. In the nucleus  $\beta$ -catenin binds to TCF/LEF transcription factors resulting in the transcriptional activation of specific target genes. The Wnt pathway has been implicated in normal SCs and homeostasis of various tissues, including the intestine and skin (38,39). The Wnt-pathway is also known to be involved in EMT (40) and therefore might have an important role in MCSCs (41). In colon cancer the Wnt pathway is known to play a central role, and CSCs could be identified using markers indicative for activity of this pathway (26). Lgr5 and CD44, both targets of the Wnt pathway could mark CSCs in intestinal and colon cancer (42,43).

The Hh pathway has been implicated in CSCs and Hh inhibition led to a reduction of CSCs in various tumors (44,45). The Hh pathway is silenced via the repression of

the signal transducer smoothened (Smo) by the transmembrane receptor Patched (Ptch). When one of the Hh ligands (Desert, Indian or Sonic) binds to Ptch, Smo repression is released leading to downstream activation of the transcription factor Gli-1, which regulates the expression of Hh target genes.

The identification of CSCs is essentially based on their ability to self-renew and to differentiate, allowing CSCs to repeatedly initiate tumors that resemble the cellular heterogeneity of the primary tumor. The use of mouse models in combination with lineage tracing experiments has proven to be effective for the identification of SCs and SC markers, which could be subsequently tested for their ability to identify CSCs in tumor models (43). Another frequently used approach to identify CSCs is the use of cell surface markers in order to isolate subpopulations of cells from patient tumor material or cell lines by FACS (46,47). The sorted cell fractions can be subsequently tested for their tumor forming potential using limited dilution and serial transplantation assays in immune-compromised mice. An alternative approach to study cells with CSC characteristics may be provided by the use of serum-free medium cell culturing known to lead to dedifferentiation of cells and acquisition of CSC characteristics (48,49). These cells often form a three-dimensional structure, called a spheroid, which has been shown to enrich for cells with SC characteristics (50,51). Other approaches to identify CSCs use specific SC properties such as high activity of specific enzymes or chemoresistance (52,53). For example, as mentioned above, the Hoechst exclusion assay is based on the enhanced activity of multidrug resistance pumps, which efflux the dye Hoechst 33342 more efficiently in SCs/CSCs. High efflux cells can be identified by FACS analyses as a typical tail, also called the Side Population (SP). SP cells showed to be more tumorigenic in mice than non-SP (32). Likewise, Aldehyde dehydrogenase (ALDH1) is an enzyme known to be highly active in cells with CSC characteristics (54). The enzyme is important for the conversion of retinol to retinoic acid (RA), RA being a major regulator of differentiation by influencing the expression of specific sets of genes. Besides selecting for CSC properties, ALDH1 positive cells also have invasive and migratory capacities (55). Assuming that a higher proportion of CSCs in a tumor correlates with poor outcome, indirect validation of potential CSC markers has been investigated by examining their expression in tumor material in relation to recurrence and survival in patient cohorts. A limitation of such studies is that cells expressing these markers are not necessarily CSCs, since only functional testing can reveal their CSC characteristics.

In EC several approaches have been used to identify CSCs, however, with little success thus far. For example, the cell surface glycoprotein and Wnt-target gene

CD44 is associated with CSCs in various solid malignancies (42,48). High expression of CD44 has been found in ESCC and EAC cell lines with CSC characteristics, such as enhanced colony formation, radiotherapy resistance and increased tumor growth in vivo (56,57). Grotenhuis et al. detected tumor-initiating cells at low frequencies of around 1:64.000 cells in patient-derived EAC samples in in vivo serial transplantation assays, however, these CSCs could not be enriched using a panel of presumed CSC markers, including CD44 and CD24 (47). The expression of several known SC or CSC markers, such as ALDH1, BMI1 and SOX2, has been determined in esophageal cancer patients' cohorts to investigate their relation with survival and recurrence, but often in a limited number of patients or only in ESCCs patients (58-60). Because EAC is the most common histological subtype in Western countries more insights in the value of possible CSC markers in EAC is important.

A better understanding of esophageal carcinogenesis and more insight in the molecular mechanisms that drive disease progression is important to optimize current treatment modalities and to develop novel targeted therapeutics. In this respect, the possible role of CSCs in esophageal cancer is of interest. Therefore, the aim of this thesis was to improve the outcome of esophageal cancer patients by optimizing current treatment modalities and investigating possible new prognostic markers. In addition the role of CSCs in esophageal cancer using a cell line based spheroid model was investigated.

### **Outline of the thesis**

Current standard treatment for esophageal cancer is a trimodality treatment consisting of neoadjuvant chemoradiotherapy (CRT) and surgery. After intended curative resection, histological examination of the resected specimen by an experienced pathologist is important to determine the microscopic radicality (R0) of the resection. Microscopic involvement of the longitudinal resection margins (R1) is a known independent prognostic factor for a poor survival. Involvement of the circumferential margin (CRM) also has been shown to be an important independent prognostic factor in esophageal cancer (61). However, the definite cut-off point has not been determined yet. In **chapter 2** we investigated the role of the CRM margin on prognosis and determined the optimal cut-off point in a relatively large patient group.

In the treatment of esophageal cancer patients who are not eligible for surgery, either due to technical irresectability or relative high comorbidity, definitive chemoradiotherapy (dCRT) is the preferred option. The two most often used chemotherapeutic regimens are: cisplatinum/ 5-fluorouracil (5-FU) and carboplatin/

paclitaxel. Although current guidelines advise cisplatin/ 5-FU as the treatment of choice (22,62), no prospective randomized study yet compared the difference in survival outcome and toxicity profiles of both dCRT regimens. Carboplatin/paclitaxel is also often used in the neoadjuvant setting and is suggested to have a favourable toxicity profile (63). Therefore, in **chapter 3** we retrospectively analyzed survival outcome and toxicity rates between the two dCRT regimens in esophageal cancer patients treated in five centres in the Northeast Netherlands.

In esophageal cancer the CSC model remains yet to be confirmed. In EAC and ESCC several markers have been associated with tumor cells with CSC characteristics (56,64,65), however not consistently and a clear signature has not yet been defined. In **chapter 4** we investigated the potential of two spheroid cultured EAC cell lines to enrich for CSCs using in vitro and in vivo assays, and to identify pathways associated with CSCs by transcriptional profiling.

Currently, no prognostic markers in BE are known that indicate progression to malignancy. GATA6 is a transcription factor associated with several gastrointestinal malignancies and suggested to have an important role in activating the Wnt pathway in pancreatic cancer (66,67). GATA6 gene amplification also has been associated with the progression of BE towards EAC and has been implicated as a prognostic factor for EAC (68,69). However, the expression of the GATA6 protein during the various stages of progression towards EAC and its relation with survival outcome in EAC patients is not known. Therefore, in **chapter 5** the expression of GATA6 in all stages of BE to EAC development and its relation with survival outcome in EAC patients was further explored

In chapter 6 we hypothesized that if CSCs are responsible for tumor recurrence, presumed CSC markers might predict survival outcome in EAC patients and could provide new prognostic markers. Several proteins have been implicated to mark CSCs in gastrointestinal cancers. Axin2 and CD44, both targets of the Wnt pathway have been associated with CSCs in colon cancer (42,70). ALDH1, Bmi1 and SOX2 are proposed CSC markers in several cancers, including intestinal and pancreatic cancer (54,71,72), but their expression in relation to patient outcome has not been investigated as yet in EAC. **Chapter 6**, describes the expression of some of these presumed CSC markers, including ALDH1, Axin2, Bmi1, CD44 and SOX2 that were determined in EAC patients treated with surgery alone, and the expression rate was related to clinicopathological features and survival outcome.



With current neoadjuvant CRT followed by standard surgical procedures, patients can be divided in two clinically relevant pathologic subcategories of responders versus non-responders. This raises questions about the necessity of surgery in those patients with pathologic complete response, if we can identify factors that can predict response to therapy and survival outcome. Several markers, such as Sonic-Hedgehog (SHH), have been proposed as predictive marker for neoadjuvant CRT in esophageal cancer patients (73). In **chapter 7** the expression CD44, SHH and SOX2 was investigated in a cohort of esophageal cancer patients treated with neoadjuvant CRT.

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